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14. ABSTRACT: We proposed to investigate the role of BMI1 (a member of polycomb gene family) in human prostate cancer (CaP) development. Here, we present the work accomplished during the final phase of the project. During the 1st phase of study we established the relevance of BMI1 in the growth and proliferation of normal and malignant prostate tumor CaP cells. In the second phase (2nd annual report), we investigated the mechanistic basis of the role of BMI1 in human CaP and established the proof of principle for BMI1 as a relevant target for CaP therapy. As provided in the 2nd annual report, we showed the data generated from studies where we employed relevant cell-based models, gene modulation techniques (such as micro-array and PCR array) and animal models. These studies showed that BMI1 sustains the proliferation of chemoresistant CaP cells (during and after chemotherapy) by regulating the expression and activity of cyclin D1 (Wnt target) and Bcl-2 (Sonic Hedgehog-SHH target). The novel finding in presented in the 2nd annual report was that regulation of Bcl-2 expression by BMI1 is independent of SHH activity in CaP cells. We showed that BMI1 regulates BCl2 by inducing the transcriptional activity of TCF4, an important executioner of β-catenin signaling pathway. In previous reports, we showed that Cyclopamine (SHH inhibitor) which is known to reduce Bcl2, fails to inhibit the growth of chemoresistant CaP cells. However, targeting of BMI1 sensitizes such chemoresistant CaP cells to Cyclopamine (SHH inhibitor) therapy. Therefore, we hypothesized that targeting of BMI1 could be an ideal strategy to sensitize hard-to-treat CaP cells for chemotherapies. In this report, we show that targeting of BMI1 by gene-silencing improved the outcome of Sulindac (Wnt-signaling inhibitor) therapy in animal models bearing prostatic tumors. We suggest that BMI1 could be exploited as a potential molecular target for therapeutics to treat chemoresistant tumors.

15. SUBJECT TERMS
BMI1, Wnt Signaling, Bcl-2, TCF, Prostate Cancer

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Introduction

Prostate cancer (CaP) is the most common visceral cancer diagnosed in men; it is the second leading cause of cancer related deaths in males in the United States and the Western world (1). Prostate cancer (CaP) patients (30-50%) will have a local or distant recurrence of disease after surgery or radiation therapy (2-4). Although castration is a common treatment option for metastatic CaP, it does not significantly prolong the survival of patients and majority of these patients progress to castration-resistant prostate cancer (CRPC). A treatment option for CRPC is cytotoxic chemotherapy; however, chemotherapy improves overall survival in such patients by only a median of 2.9 months (4). Despite chemotherapy, CRPC patients typically show rapid progression and develop chemoresistant disease (4-5). Therefore emergence of chemoresistance is considered a major hurdle in the management of CaP. The dismal outcome of the management of chemoresistant CRPC disease could also be associated to the lack of knowledge about the molecular mechanism involved in the development of chemoresistant disease.

There is increasing evidence that polycomb group (PcG) proteins, first discovered in *Drosophila* as epigenetic gene silencers of homoeotic genes, play a crucial role in cancer development and disease recurrence (6). BMI1, a member of PcG family of repressor proteins, is a well-known marker used in stem cell biology (6-7). There is an enormous body of evidence suggesting that increased expression of BMI1 could facilitate chemoresistance in solid tumors (6-7). Recent studies show that BMI1 is positively correlated with poor prognosis in cancer patients (8-11). We recently reviewed the significance of BMI1 in the emergence of chemoresistance in various types of cancers (6). Glinsky *et al* identified BMI1 as one the signature molecules in a broad spectrum of therapy-resistant cancers included CaP (12). Except a few regulatory functions of BMI1 in cell cycle (suppressing p16INK4a and p14ARF), not much is known about the mechanism of action of BMI1. In this current study, we determined the relevance of BMI1 in the chemoresistance of CaP disease and delineate its mechanism of action both *in vitro* and *in vivo*. In addition, we establish the utility of BMI1 as a molecular target for therapeutic agents to overcome chemoresistance.

Body

Under this section we provide information about the experimental and materials and methods used to accomplish our objectives as stated in the proposal.

Experimental Design for Specific Aim #1

We conducted the experiments to define the effect of overexpression and silencing of BMI1 gene in CaP cells. For this purpose, we (a) knockdown the BMI1 gene by transfection of siRNA and (b) overexpressed the BMI1 gene by transfecting BMI1 construct (pbabe-BMI1 plasmid provided by Professor Chi Van Dang, Professor of Cell Biology, School of Medicine, The Johns Hopkins University, Baltimore, MD) in PC3 (androgen-independent), LNCaP (androgen-dependent), 22Rν1 (androgen-sensitive) and normal prostate epithelial cells (PrEC) cells. We then studied the growth and viability of transfected cells *in vitro* by employing the MTT assay. To investigate the effect of BMI1 gene on the rate of proliferation of CaP cells, we employed ^{3}[H]thymidine uptake assay. This assays measures the amount of ^{3}[H]thymidine taken up by dividing cells (for DNA synthesis) thus gives a measure of the rate of division or proliferation of cells. BMI1 silenced and BMI1 overexpressing CaP cells were cultured in presence of ^{3}[H]thymidine and ^{3}[H] thymidine uptake was measured by Liquid scintillation counter. These cells were also measured for DNA content. Since BMI1 was observed to increase the proliferative potential of CaP cells and to establish that BMI1 indeed was a driving force for proliferating cells, we investigated whether BMI1 has to potential to drive proliferation of normal prostate epithelial cells. For this purpose, BMI1 was overexpressed in normal prostate epithelial cells (PrEC). We chose PrEC cells because under normal culture conditions, PrEC cells are known to replicate between 3-4 cycles and after 4 cycles, these cells enter

into a mode of senescence. The break of senescence in normal epithelial cells is a hall mark of progression towards proliferation. As a control to study, another set of PrEC cells were transfected alone vector (pbabe). Further a microarray was performed with BMI1 silenced LNCaP cells to understand the mechanism of action of BMI1 in CaP cells. Experiments conducted under this aim provided information whether genes involved in proliferation are regulated by BMI1 gene. These data were validated by western blot analysis. We analyzed the expression level of Cyclin D1, p16 and Bcl-2 protein in CaP cells. Next we investigated whether the overexpression generates the data contrary to what was observed in BMI1 silenced cells. For this purpose BMI1 was overexpressed in LNCaP, PC-3 and DU145 cells by transfecting pbabe-BMI1 plasmid. Cell lysates prepared from these cells were analyzed for Cyclin D1, Bcl-2 and p16 proteins by employing western blot analysis. To understand the mechanism through which BMI1 regulates Cyclin D1, we carried out experiments on critical pathways which are already known to be associated with Cyclin D1 expression. This includes Wnt/β-catenin signaling pathway. We asked whether BMI1 has any association with Wnt/β-catenin signaling (which is itself reported to control Cyclin D1). Interestingly, we found that BMI1 overexpression causes an increase in the transcriptional activation of *TCF*-responsive element (a bio-marker of Wnt signaling) in CaP cells. Since Bcl-2 was observed to be modulated by BMI1, we investigated if BMI1 has any association with sonic hedgehog (SHH) pathway that is very well know to regulate Bcl-2. For this purpose we determined the expression level of Bcl-2 in BMI1-overexpressing and BMI1-silenced CaP cells in presence of Cyclopamine, a SHH pathway inhibitor. We also tested if re-introduction of BMI1 would restore the Bcl-2 levels in CaP cells pre-treated with cyclopamine (SHH inhibitor). Further, we investigated an association of tcf and Bcl-2 in CaP cells. We investigated the mechanism through which BMI1 drives the Tcf/Bcl-2 signaling in CaP cells.

Experimental Design for Specific Aims #2 and 3

Animal studies showed a significant lower tumor growth in PC-3-empty vector and PC-3-BMI1-supressing cell-originated tumors than PC-3-BMI1-overexpressing cell originated tumors in athymic mice. We showed that knocked down of BMI1 sensitized the chemoresistant prostatic tumors for the Docetaxel and sulindac therapies.

Material and Methods

Cell Lines and plasmids: Primary prostate epithelial cell (PrEC) was procured from Cambrex BioScience (Walkersville, MD). Normal prostate cell line **(**RWPE1), CaP cell lines (LNCaP, PC3 and Du145), and colon cancer cell line HT29 were obtained from ATCC (Manassas, VA). pGeneClip-BMI1-shRNA plasmid was procured from SA-Biosciences (Fredrick, MD). pTK-TCF-Luc (TopFlash & FopFlash) was procured from Millipore (Temecula, CA).

Chemicals and reagents: Docetaxel and Sulindac were purchased from LKT Laboratories (St. Paul, MN). Puoromycin, G418 and BrdU labeling reagent were purchased from Invitrogen (Carlsbad, CA). The anti-BMI1 antibody, anti-TCF4 antibody was obtained from Millipore. Anti-BCL2 and anti-cyclin D1 antibodies were purchased from Cell Signaling (Danvers, MA).

Western blot Analysis. This was performed as per method described earlier (13-15). Briefly, cell lysates were prepared by incubation of cells for 30 min in ice-cold lysis buffer [(0.05 mmol/L Tris-HCl, 0.15 mmol/L NaCl, 1 mole/L EGTA, 1 mol/L EDTA, 20 mmol/L NaF, 100 mmol/L Na_3VO4, 0.5% NP-40, 1% Triton X-100, 1 mol/L phenyl methylsulfonyl flouride (pH 7.4)] with protease inhibitor cocktail (Roche, Indianapolis, IN). The lysate was collected; insoluble materials were removed by centrifugation at 4°C for 15 minutes at 15,000g, and stored at -80 °C. BCA protein estimation kit was used to estimate the protein concentration in the lysates (Pierce, Rockford, IL), as per the vendor's protocol. Next, 40 µg protein was resolved in 10% SDS-PAGE gels, transferred onto PVDF membranes (Millipore) and incubated in blocking buffer (5% nonfat dry milk/1% Tween 20; in 20mmol/L TBS, pH 7.6) for 2 h. The blots were incubated with appropriate primary antibody, washed

and incubated with HRP-conjugated secondary antibody (Sigma). The blots were detected with chemiluminescence (ECL kit, Amersham Biosciences, Piscataway, NJ). Equal loading of protein was confirmed by stripping the blots and re-probing with β-actin (Sigma).

***Tumor studies*:** Athymic (nu/nu) male nude mice (6 weeks old; HarlanTek, Madsion, WI), were implanted with PC3 cells (1 x 10^6) in 50 µl RPMI + 50 µl Matrigel (BD Biosciences, Bedford, MA) subcutaneously into the right flanks of each mouse. At 7th day post-implantation, the study was divided into three protocols.

Stable transfection- BMI1-shRNA-mediated Silencing protocol. Group-1 (n=10) of mice implanted with empty-vector (pGenCLIP) transfected stable cells and treated with vehicle served as control. Group-II (n=10) included mice implanted with vector transfected cells and treated with docetaxel (10 mg/kg). Group-III (n =10) included mice implanted with BMI1-silenced tumor cells and received *i.p.* administration of saline. Group-IV (n = 10) included mice implanted with BMI1-silenced tumor cells and received docetaxel treatment.

SiRNA-treatment mediated BMI1-silencing protocol : *Group I* of mice served as control group and recieved 0.1. ml of corn oil and scrambled siRNA in 0.1ml of liposomes through intraperitoneal (i.p.) route. *Group II* of mice received Sulindac (50 mg/kg in 0.1 ml of corn oil *i.p*.) and scrambled siRNA in 0.1 ml of liposomes (3-times/wk, i.p). *Group III* mice were treated with BMI1-siRNA (0.8mg/kg ; 3-times/week in 0.1 ml liposomes) and Sulindac (50 mg/kg; 3-times/week).

Tumor measurement: Body weights were recorded seven days/week throughout the study. Tumor growth was recorded as described (13-15). Tumors from three animals from control and treated groups were excised at the 35th day post administration when 100% of control animals reached the preset end point of tumor volume of 1,000 mm^3 (for stable transfection protocols) and 500 mm^3 (for BMI1-siRNA treatment protocol). Rest of the animals in other groups remained under protocol for a maximum time of 10 weeks. Before 2 h of sacrifice, each animal received an *i.p.* administration of BrdU labeling reagent (10 ml/ kg) to label proliferating cells within tumors (13). All procedures conducted were in accordance with the IACUC guidelines.

***Statistical analyses*:** Student's *t test* for independent analysis was applied to evaluate differences between the treated and untreated cells with respect to the expression of various proteins. A Kaplan-Meier survival analysis with the corresponding Log-Rank and Linear Regression analysis was used to measure the rate of mean tumor volume growth as a function of time. A p-value of < 0.05 was considered to be statistically significant.

Key Research Accomplishments in the report

Under the final phase of the study, we accomplished goals as proposed under tasks 3 (provided in the Statement of Work-SOW). These are described as following:

Task 3B : **Studies in athymic nude mouse xenograft model will be conducted (a) to analyze the consequences of BMI1 silencing on tumorigenicity of human CaP cells, and (b) to evaluate the effect of BMI1 siRNA in combination with chemotherapies *in vivo*.**
Status: Data presented in this report.

Results

Targeting of BMI1 sensitized human prostatic tumors to Docetaxel and Sulindac chemotherapies in xenograft mouse models:

Our previous studies showed that BMI1-overexpressing tumors exhibit hard-to treat character, which is generally termed as chemoresistance. We next asked if targeting of BMI1 could sensitize the CaP tumors for clinically used chemotherapies. For this reason we employed two approaches. Under first

approach, we rendered CaP cells BMI1-suppressed by a stable transfection technique.
Under the 2nd approach, we used a continuous gene therapy technique to suppress the BMI1 expression in tumors. Mouse under protocols such as BMI1-suppression protocol and gene therapy protocol were exposed to chemotherapies.

Rationale for selection of chemotherapeutic agents: The selection for chemotherapies was performed on the basis of their relevance to clinical use and the signaling pathways identified as targets of BMI1 in the current study. For the BMI1-suppression protocol, we selected Docetaxel as a chemotherapeutic agent because it is widely used in clinics to treat metastatic CaP in men. Approved by the US Food and Drug Administration (FDA) in 2004 for clinical use, docetaxel remain a mainstay of therapy for CRPC patients. However, resistance to docetaxel is a significant clinical problem given that 50% men suffering from CRPC exhibit poor or no responsiveness to therapy. The problem is further thus compounded from the clinical observations that patients who initially respond to therapy ultimately develop resistance to docetaxel. Therefore improving treatment outcomes for patients with docetaxel resistance is a high priority because of the limited number of treatment options historically available to this group of patients.

For the gene-therapy protocol, we selected Sulindac, a well-known inhibitor of Wnt/β-catenin signaling. Our mechanistic data showed that BMI1 control Bcl2 expression in chemoresistant CaP cells by a regulating activity of TCF4-transcriptional factor. We also observed that BMI1 regulates the binding of TCF4-transcriptional factor on the promoter region of *BCL2 gene* in CaP cells. TCF-4 transcriptional factor is the final molecule that relays Wnt signaling, and acts as the final executor of this important signaling pathway in tumor cells. Therefore, we speculated that targeting of Bcl2 expression at two critical upstream stages viz., (i) BMI1 (which is the master controller) and (ii) TCF-4 (which relays signal from BMI1 and Wnt), will be an ideal approach to inhibit growth of CaP cells, particularly chemoresistant phenotype.

BMI1-suppression by stable transfection and Docetaxel therapy: BMI1-supressed cell-derived tumors were observed to grow at slower rate than control tumors (Fig. 1A). This was evident from the significant difference in the rate of growth and tumor volumes between control and BMI1-supressed group of animals (Fig. 1A). Mice implanted with control tumors reached a preset end-point tumor volume of 1000 mm^3 at 49th day of post-implantation (Fig. 1A). It is noteworthy that the average volume of tumors in mice bearing BMI1-silenced tumors did not reach the end-point even at 70th day post-implantation (Fig. 1A). At 49th day, control group of animals treated with docetaxel exhibited an average tumor volume of 850 mm^3. However at this point, BMI1-silenced group of animals treated with docetaxel exhibited an average tumor volume of 230 mm^3 suggesting that BMI1-silencing sensitizes tumor cells to docetaxel therapy (Fig. 1A). Next, we evaluated whether docetaxel caused a delay in the growth of BMI1-suppressed tumors. The observed differences between control and BMI-silenced group of animals were statistically significant ($p<0.05$, Fig. 1B).

BMI1-suppression by siRNA administration and Sulindac therapy: Our observations in BMI1-suppression protocol showed that targeting of BMI1 significantly decrease the growth of prostatic tumor implanted in mice (Figure 2A-B). This established the proof of principle that BMI1 targeting is an ideal approach for CaP treatment. However, BMI1 suppression by transfection is not possible in patients at clinics. Therefore, we hypothesized that targeting of BMI1 by gene therapy "with the use of siRNA-oligos" is practically possible. If successful, it would have high translational relevance.

Study Design: To test our hypothesis, we performed a 5-week treatment protocol study in athymic male mice and set 500 mm^3 tumor volumes as a preset-endpoint. Mice were implanted with PC-3 cells (1×10^6) cells and allowed to grow tumor for 1-Week. 100% of mice exhibited visible tumors after 1 week of implantation. At this stage mice were randomly divided into three (3) groups. Group 1

of mice receiving intraperitoneal (i.p.) administration of vehicle (corn oil + liposomes) alone (0.1.ml/3 time a week) served as control. The vehicle (liposomes) is a commercially available and tested vehicle designed to deliver oligos *in vivo*. The control mice exhibited increase in tumor growth as a function of time and majority of mice reached the preset-end-point at 5 week post-treatment. The second (2^{nd}) group of mice was treated mice with Sulindac (50 mg/kg in 0.1 ml corn oil; 3-times a week). This group also received scrambled siRNA in liposomes (0.1. ml; 3-times/week). The third (3^{rd}) group of mice received Sulindac (50mg/kg; 3-times a week) and BMI1-siRNA in liposomes (3-times/week).

Outcome: As compared to control mice, Sulindac treatment substantially decreased the growth of tumors in mice (Fig. 2A). At 5^{th} week when average tumor volume in control group was 773± 78 mm^3, (mean +SE) the Sulindac-treated group (2^{nd} group) exhibited an average tumor volume of 446 ± 24 mm^3 (mean +SE) (Fig. 2A). It is noteworthy that mice receiving combination treatment (3^{rd} group) exhibited increased inhibition (almost 80% inhibition) in growth of tumors and at 5^{th} week-post treatment, exhibited an average tumor volume of 100 ± 16 mm^3 (mean +SE) (Fig. 2A).

We next measured the average weights of the tumors excised from the mice of all group. It is noteworthy to mention that all mice received equal number of cells at the time of implantation, and treatments were started began when all mice were randomly distributed in groups after one of implantation (when all mice exhibited visible tumors). Therefore, it could be now ascertained that tumors grew as a function of time and tumor weights exhibited by different were indeed influenced by treatments. At the termination of study, the average tumor weight were in (a) 525 ± 35 mg (mean ± SE) in control group, (b) 236 ± 13 mg (mean ± SE) in Sulindac-treated group and (c) 98 ± 11 mg (mean ± SE) in combination group (BMI1-siRNA + Sulindac) (Fig. 2B).

Effect of BMI1-siRNA monotherapy and Sulindac-based combination (Sulindac +BMI1-siRNA) on cell proliferation and Bcl2 expression in tumors: We determined significance of BMI1 in the proliferation of cells within tumors exposed to Sulindac therapy. Proliferating tumor cells are known to express PCNA protein in tumor cells therefore provides an indirect measure of *in vivo* proliferation. We analyzed tumor samples harvested from animals exposed to sulindac, BMI1-siRNA and combination therapy (BMI1-siRNA +Sulindac). Notably, tumor sections (harvested at 5^{th} week-post treatment) showed that Sulindac treatment decreased the PCNA expression in tumors (Fig. 3). BMI1-silenced tumors exhibited decreased BCL2 levels (Fig. 3). However, the effect of combination on Bcl2 expression was profound (Fig. 3).

Effect of BMI1-siRNA monotherapy and Sulindac-based combination (Sulind +BMI1-siRNA) on nuclear TCF-4 levels in tumors: We next determined the effect of mono and combinational therapies on the expression of TCF4 expression levels in the nucleus of tumor cells. Tumor sections (harvested at 5^{th} week-post treatment) showed that Sulindac treatment decreased the TCF4 expression in tumors (Fig. 4). BMI1-silenced tumors exhibited decreased TCF4 nuclear levels (Fig. 3). However, the effect of combination on nuclear levels of TCF4 was profound (Fig. 4).

Effect of BMI1-siRNA monotherapy and Sulindac-based combination (Sulindac +BMI1-siRNA) on Cyclin D1 levels in tumors: We next determined the effect of mono and combinational therapies on the expression of Cyclin D1expression levels in tumor cells. BMI1-silenced tumors exhibited decreased Cyclin D1 expression levels (Fig. 4). However, the effect of combination on nuclear levels of Cyclin D1 was profound (Fig. 4). These data show that BMI1 confers chemoresistance to prostatic tumors, and abolishing BMI1 sensitizes chemoresistant tumors to chemotherapy, therefore establishes its significance as a therapeutic target.

Reportable Outcomes

Based on our results, three major observations were found to be reportable. These observations were submitted for their publication in scientific journals.

On the basis of our studies, following major observations were made:

1. BMI1 protein levels progressively increase with the advancement of CaP disease in humans. In addition, BMI1 protein is increased in disease which is resistant to therapy. The significance of this outcome is that tissue biopsies in future could be analyze for BMI1 protein to assess if the disease would be aggressive and treatable or not. BMI1 would act as a future biomarker. This would save time of clinicians to manage the CaP disease in men. This is an important reportable outcome.
2. The significance of this study is that chemoresistant prostatic tumors could be treated now by targeting BMI1. This study open up the opportunities to develop new drugs and therapies those specifically could target BMI, thus could be used to overcome therapy resistance of hard-to treat tumors. This is an important outcome of this study.

Conclusion

Recent studies showed that dysregulation of BMI1 alters cell proliferation, senescence and self-renewal of several human cancers (6,10). It is speculated that inability of tumor cells to undergo apoptosis in response to chemotherapy results in a selective advantage for such tumor cells to become more aggressive compared to chemoresponsive cells during progression of CaP (6). Several studies demonstrate that BMI1 rescues tumor cells from apoptosis and could be a critical factor involved in the emergence of chemoresistance, however no concrete mechanism of action is yet known (8-10). Chemoresistant CRPC is hard-to-treat disease and identifying a critical molecule that confers the chemoresistant characteristic to such tumors would be an important advancement in the field of cancer therapy. In the current study, we provide mechanism-based evidence to show that BMI1 plays a critical role in deciding the therapeutic outcome and the fate of tumor cells undergoing chemotherapy. This study is significant because we demonstrated that BMI1 equally confers chemoresistance to hormone-sensitive CaP and CRPC cells. This is further strengthened by the data that BMI1 expression does not get influenced by androgen. Our data is significant because it explains the possibility of BMI1 as a part of the mechanism that drives indolent disease to aggressive phenotype which is often androgen-independent. This observation carries high significance because CRPC tumors in men proliferate under low androgen conditions (5). Based on our data we suggest that targeting BMI1 should be a part of strategy when therapeutic plans are devised to combat chemoresistant type of cancer.

One of the important observations of this study is that BCL2 and Cyclin D1 (found to be regulated by BMI1) have a commonality to also be functional members of Wnt and Shh pathways (16-20). Activity of BCL2 and Cyclin D1 are reported to be high in chemoresistant tumors (13,22-24). Keeping in view the critical role of BCL2 in chemoresistance, targeting the protein directly (anti-BCL2 immunotherapy) or blocking the pathways (such as Shh) which regulates its expression, is being suggested as an ideal strategy to overcome chemoresistance of tumor cells (21, 25-26). Shh inhibitor (Cyclopamine) known to downregulate BCL2 in some tumors is currently being investigated as a therapeutic agent for basal cell carcinoma, medulloblastoma, rhabdomyosarcoma, and glioblastoma (27). However the mechanism that causes the resurgence of tumor cells after BCL2-targeted therapy is not known. Our study is significant because we show that BCL2 is not completely lost in tumor cells after chemotherapy and alternate pathways (such as BMI1/TCF4 molecular pathway) regulate BCL2 in chemoresistant tumor cells. This is based on our data showing that (i) BMI1 regulates BCL2 independent of Shh-signaling in chemoresistant tumor cells and (ii) elevated levels of BMI1 and BCL2 in cells those survived chemotherapy. We suggest that this mechanism could be an explanation for the survival of chemoresistant cells post-chemotherapy. Although the previous report showed that BMI1 itself is a target of Shh-signaling, our data show that BMI1 acts independent of Shh

(28). It is possible that chemoresistant cells expressing BMI1 are a highly selected sub-population that remains hard to treat and play an important role in indolence of disease in human CaP patients. BMI1 activity is manifested in the form of repression of target genes such as p16 and the mode of action could be through epigenetic silencing, modulation in the methylation states of genes.4 However, in this study we observed that BMI1 upregulates *BCL2* gene. Keeping in view the repressive nature of BMI1, there was a need to understand the mode of action (other than repression) through which BMI1 induces BCL2 expression and activity. We provided evidence that BCL2 activation in chemoresistant cells under the guidance of BMI1 is mediated by TCF4 in tumor cells. This was validated in prostate and colon cancer cells *in vitro*; and in human prostatic tissues. We identified the binding regions of TCF4 transcriptional factor on the promoter of *BCL2*. By conducting several ChIP assays, we observed that binding efficiency of TCF4 to the *BCL2*-promoter is dependent on the BMI1 levels. Although the complete information about the regulation of TCF4 by BMI1 is not completely understood, current data suggest that TCF4 indeed is in part under the control of BMI1. The significance of our data is that it (*i*) identifies BMI1-induced TCF4 as a molecular module that drives Wnt-signaling within chemoresistant tumor cells, and (*ii*) *BCl2* as a target of BMI1/TCF4 molecular module. Based on our data, we speculate that molecular module could be operational during emergence of chemoresistance in CaP cells and also responsible for the survival and proliferation of chemoresistant tumor cells after chemotherapy.

Docetaxel has been tested under several clinical trials alone and in combination with other agents to treat CaP. Docetaxel therapy was observed to result in a PSA drop of more than 50% in CaP patients, an observation made in several trials such as the SWOG trial (29). However, docetaxel alone, and in combination do not completely abrogate the tumor or bring down PSA levels to the normal in human CaP patients (29). Although effective in CaP patients to an extent, some CaP conditions do not respond to docetaxel therapy and such patients do not exhibit changes in PSA level after therapy (30). In this context, this study is highly significant as we show that targeting BMI1 in chemoresistant CRPC cells sensitizes tumor cells to docetaxel therapy both *in vitro* and *in vivo*. This study identified BMI1 as an ideal molecule to be targeted to overcome the chemoresistance of CaP cells and corroborates to earlier report showing the utility of BMI1 as a target to overcome chemoresistance in ovarian cancer cells (9). Under *in vivo* conditions, the significance between BMI1-positive, BMI-silenced and BMI1-overexpressed tumor cells vis-à-vis docetaxel therapy was significant. The success of docetaxel therapy against prostatic tumors in a xenograft mouse model was observed to be highly dependent on the level of BMI1. We suggest that preventing the development of chemoresistance in CaP patients will be beneficial for a large group of patients and interventions directed against BMI1 may provide opportunities to enhance the efficacy of chemotherapy. In this direction we have opened another front by identifying small molecule inhibitors of BMI1. We suggest that these should be explored against chemoresistant tumors. The advanced work with small molecule inhibitors of BMI1 against chemoresistant tumors is underway in our laboratory.

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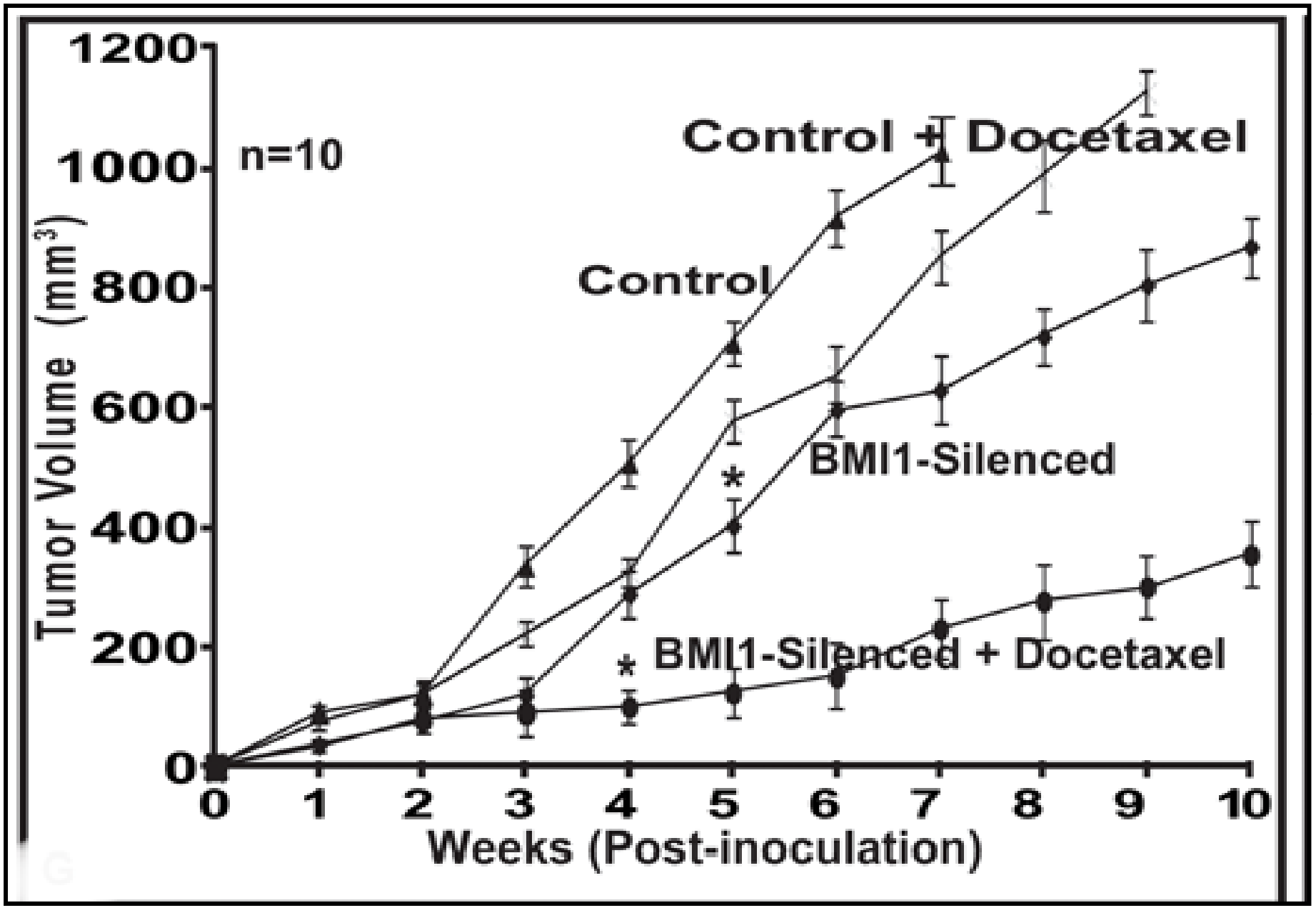
1200
1000
800
600
400
200
0
n=10
Tumor Volume (mm³)
Control + Docetaxel
Control
BMI1-Silenced
BMI1-Silenced + Docetaxel
*
*
0
1
2
3
4
5
6
7
8
9
10
Weeks (Post-inoculation)

Figure 1A

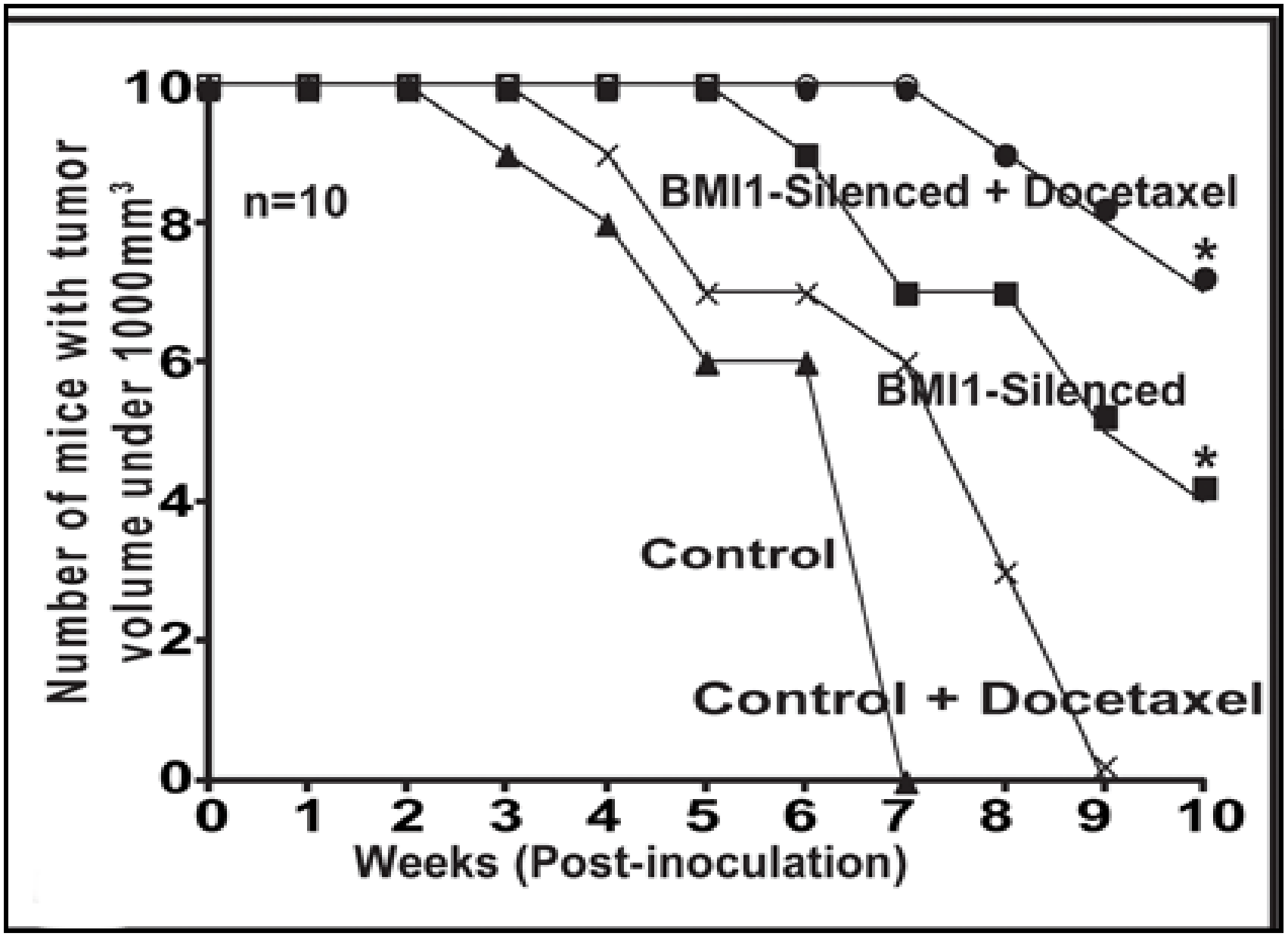
Number of mice with tumor volume under 1000mm³
n=10
BMI1-Silenced + Docetaxel
BMI1-Silenced
Control
Control + Docetaxel
*
*
0
2
4
6
8
10
0 1 2 3 4 5 6 7 8 9 10
Weeks (Post-inoculation)

Figure 1B

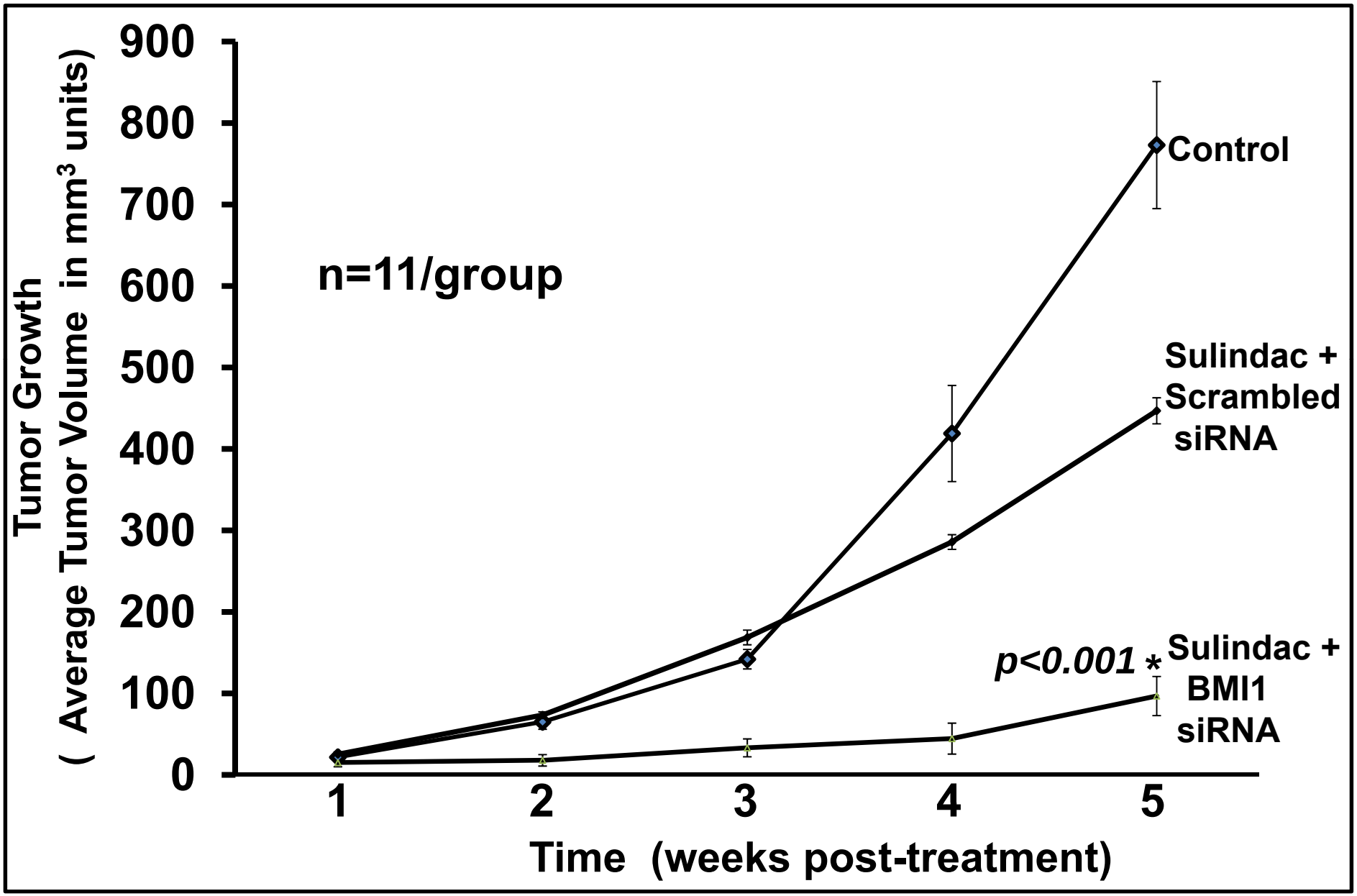
Tumor Growth
(Average Tumor Volume in mm3 units)
900
800
700
600
500
400
300
200
100
0
n=11/group
Control
Sulindac +
Scrambled
siRNA
p<0.001 *
Sulindac +
BMI1
siRNA
1
2
3
4
5
Time (weeks post-treatment)

Figure 2A

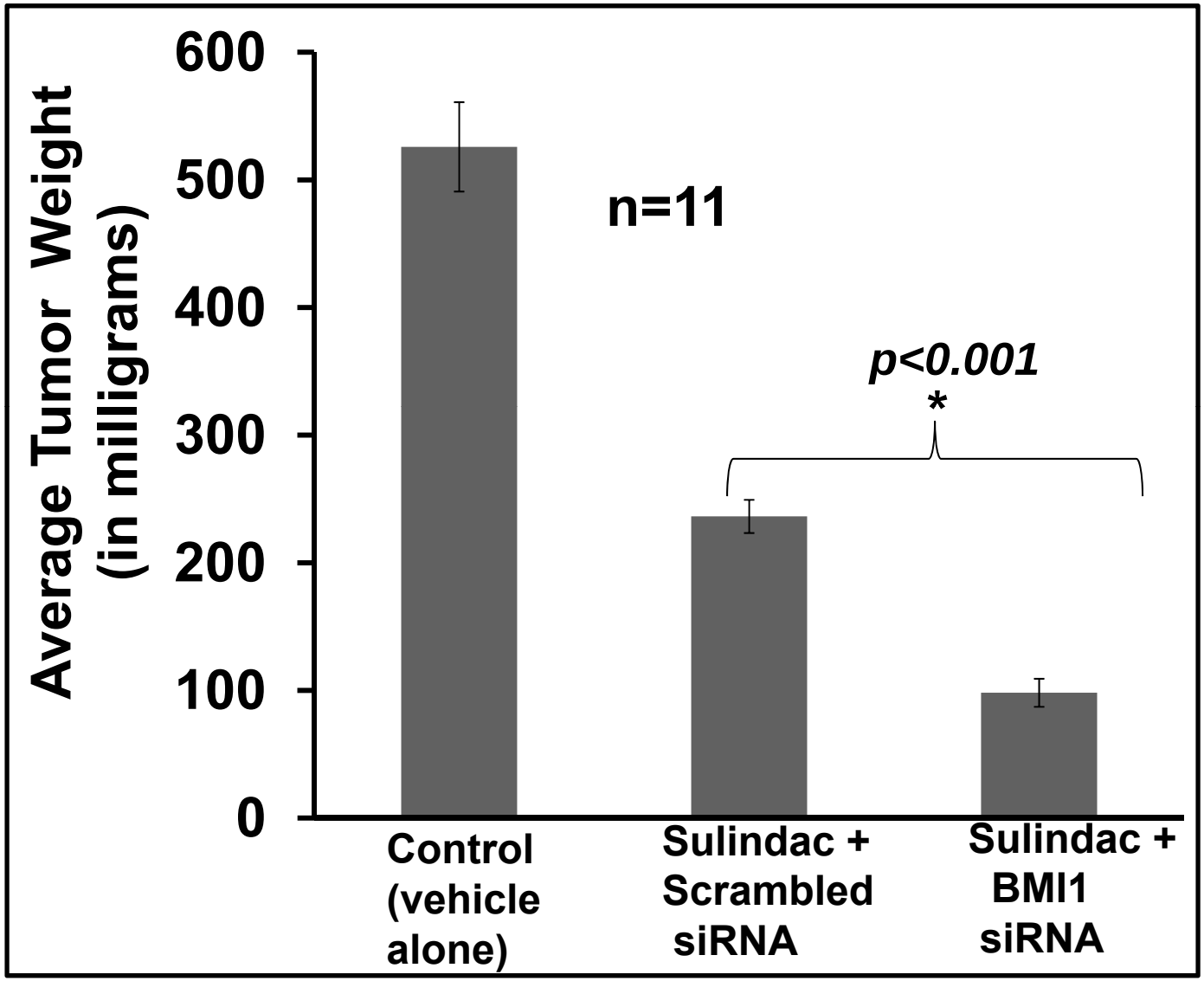
Average Tumor Weight (in milligrams)
600
500
400
300
200
100
0
n=11
p<0.001
*
Control (vehicle alone)
Sulindac + Scrambled siRNA
Sulindac + BMI1 siRNA

Figure 2B

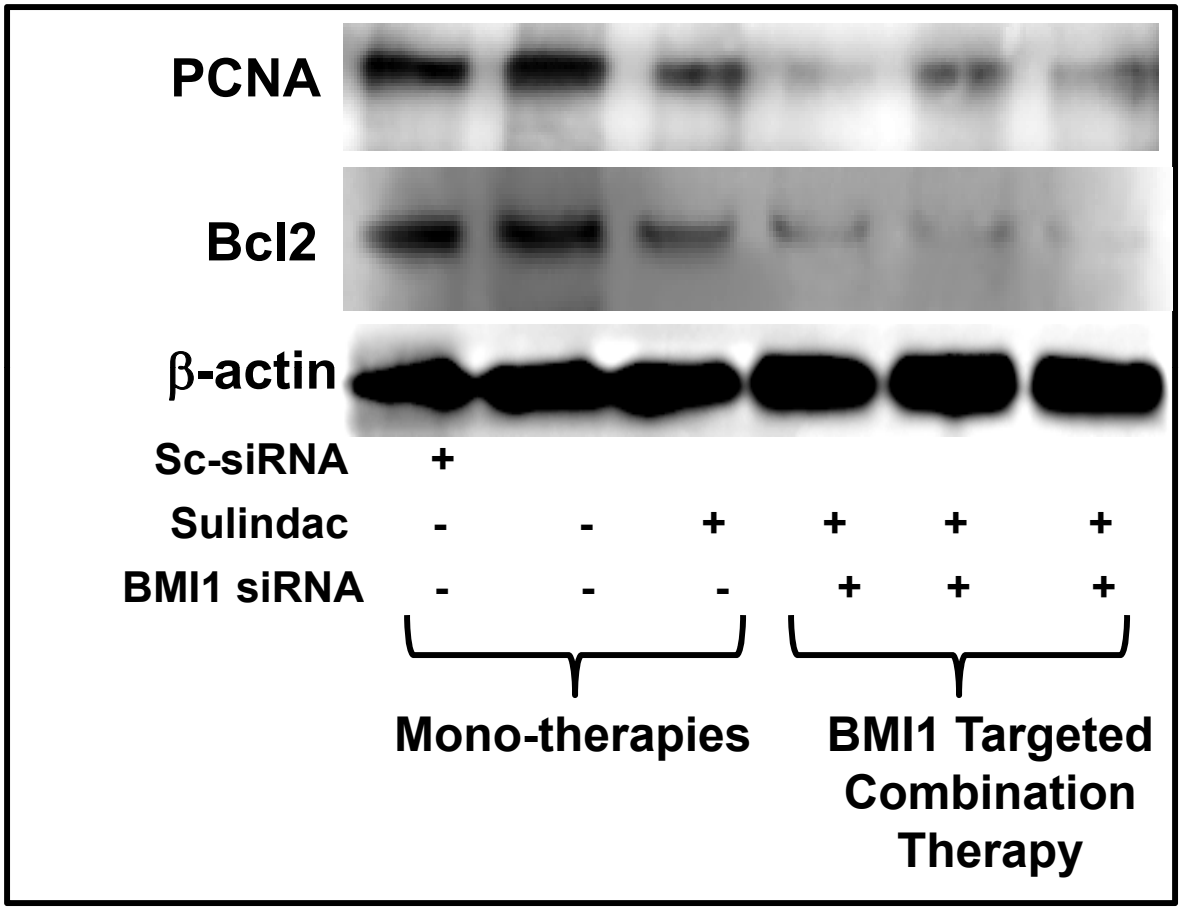
PCNA
Bcl2
β-actin
Sc-siRNA + - - - - -
Sulindac - - + + + +
BMI1 siRNA - - - + + +
Mono-therapies
BMI1 Targeted Combination Therapy

Figure 3

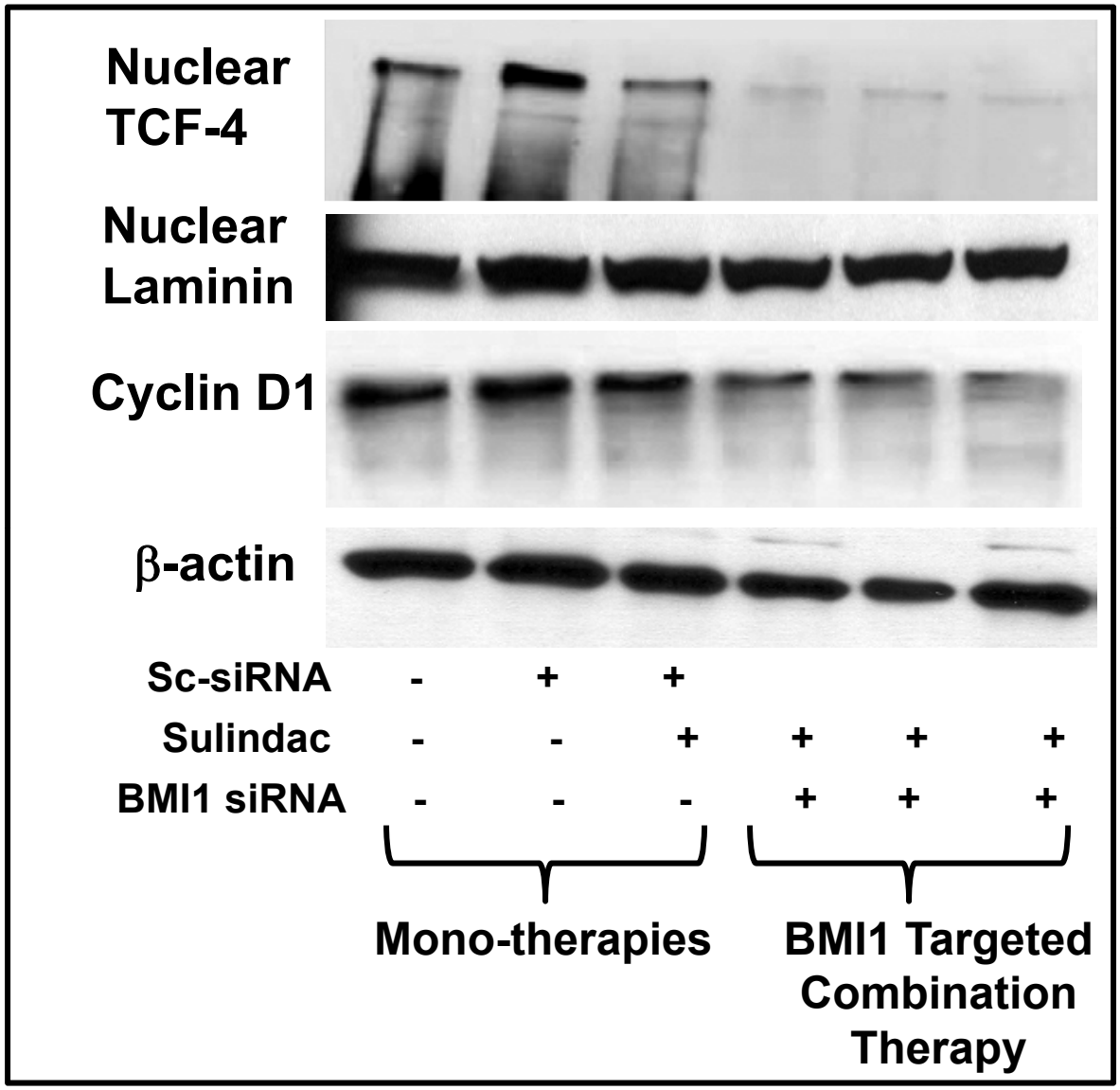
Nuclear TCF-4
Nuclear Laminin
Cyclin D1
β-actin
Sc-siRNA - + +
Sulindac - - + + + +
BMI1 siRNA - - - + + +
Mono-therapies
BMI1 Targeted Combination Therapy

Figure 4

Supplementary Figure 1

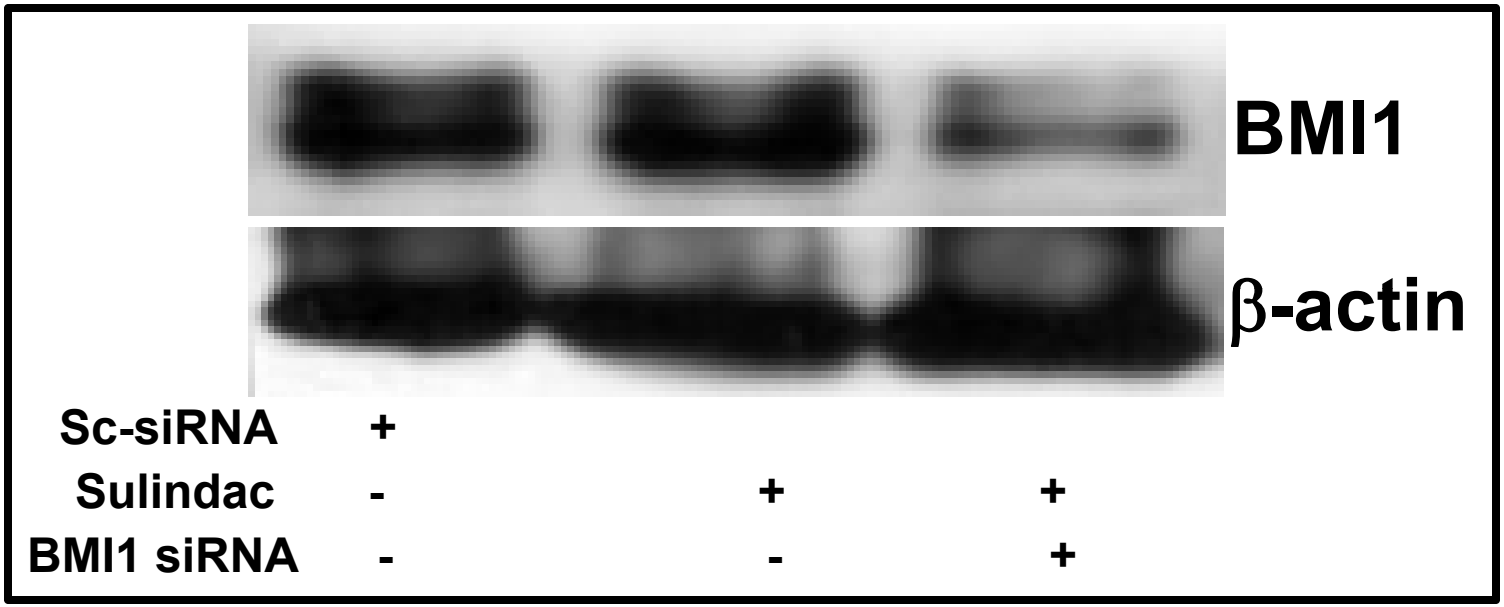

Figure legends

Figure 1. Targeting of BMI1 by gene therapy sensitizes tumors to Docetaxel chemotherapy *in* xenograft mouse models. (A) The graphical representation of data showing the effect of targeting of BMI1 in tumor cells and responsiveness to docetaxel therapy in mice implanted with PC3-derived tumor cells. The growth was measured in terms of average volume of tumors as a function of time. Data is represented as mean ± SE, * indicates $p < 0.05$ from the control group. (**B**) The graphical representation of the data showing the number of mice remain with tumor volumes <1000 mm^3 after BMI1-silencing for indicated weeks.

Figure 2. Targeting of BMI1 by gene therapy sensitizes tumors to Sulindac chemotherapy *in* xenograft mouse models. (A) The graphical representation of data showing the effect of targeting of BMI1 by siRNA delivered in liposomes (3-times/ week) in tumor cells and responsiveness to Sulindac therapy in mice implanted with PC3-derived tumor cells. The growth was measured in terms of average volume of tumors as a function of time. Data is represented as mean ± SE, * indicates $p < 0.05$ from the control group. (**B**) The bar graph shows the average tumor weights (harvested from mice at the termination of study). The details are described under Materials and Methods.

Figure 3. Targeting of BMI1 increases the chemotherapeutic potential of Sulindac (A) against the proliferation of tumor cells and (B) on BCL2 expression *in vivo.* Immunoblot images showing (**A**) PCNA expression (cell proliferation marker) and (**B**) BCL2-expression in tumors as assessed by immunoblot analysis. The immunoblotting data was confirmed in all specimens from each group. The data from three tumor specimens from combination therapy group are presented. One tumor sample each from control groups (vehicle control, scrambled siRNA, sulindac + scrambled –siRNA) was used in the image shown here.

Figure 4. Targeting of BMI1 increases the chemotherapeutic potential of Sulindac on β-catenin signaling pathway *in vivo.* Immunoblot images showing expression of cytoplasmic and nuclear β-catenin levels in tumors treated with monotherapies and BMI1-targeted combinational therapies. The data from three tumor specimens from combination therapy group are presented. One tumor sample each from control groups (vehicle control, scrambled siRNA, sulindac + scrambled –siRNA) was used in the image shown here.

Supplemental Figure 1. Image showing the effect of Sulindac and BMI1-specific siRNA on the expression of BMI1 in PC-3 cells. Immunoblot images showing expression of BMI1 in PC-3 cells treated with sulindac and BMI1-targeted siRNA.

Acknowledgements of funding in manuscripts

The funding (W81XWH-08-1-0605) from CDMRP-DOD has been acknowledged in the paper published. Published papers are attached as Appendices I.

1: Paper published in Scientific Journal "**Stem Cells**, 30: 372-78; 2012" (**Appendix I**).

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Concise Review: Role of BMI1, a Stem Cell Factor, in Cancer Recurrence and Chemoresistance: Preclinical and Clinical Evidences

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Key Words. BMI1 • Cancer stem cells • Cancer recurrence • Chemoresistance • Cancer

ABSTRACT

There is increasing evidence that a variety of cancers arise from transformation of normal stem cells to cancer stem cells (CSCs). CSCs are thought to sustain cancer progression, invasion, metastasis, and recurrence after therapy. Reports suggest that CSCs are highly resistant to conventional therapy. Emerging evidences show that the chemoresistance of CSCs are in part due to the activation of B cell-specific Moloney murine leukemia virus integration site 1 (BMI1), a stem cell factor, and a polycomb group family member. BMI1 is reported to regulate the proliferation activity of normal, stem, and progenitor cells. BMI1 plays a role in cell cycle, cell immortalization, and senescence. Numerous studies demonstrate that BMI1, which is upregulated in a variety of cancers, has a positive correlation with clinical grade/stage and poor prognosis. Although evidences are in support of the role of BMI1 as a factor in chemoresistance displayed by CSCs, its mechanism of action is not fully understood. In this review, we provide summary of evidences (with mechanism of action established) suggesting the significance of BMI1 in chemoresistance and recurrence of CSCs. STEM CELLS *2012; 30:372–378*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Traditional cancer therapies typically target the rapidly dividing tumor cells, however, some cells of the tumor are spared [1–3]. These spared tumor cells which are reported to be present within many tumor types exhibit the potential to regenerate and are called cancer stem cells (CSCs) [1–5]. This may explain the clinical scenario in which a tumor has an apparent volumetric reduction, however, is subsequently followed by local recurrence. While debate continues as to the precise identity and function of CSCs, there is general agreement that CSCs display increased chemoresistance and radioresistance [1-3, 6]. Therefore, understanding the biology of chemoresistance potential of CSCs may contribute to our understanding of tumor biology and would have far-reaching clinical implications. Although several molecules have been reported to confer chemoresistance to CSCs, much is not known whether stem cell factors play a role in chemoresistance of tumor cells including CSCs.

There is increasing evidence that polycomb group (PcG) proteins (discovered in *Drosophila* as epigenetic gene silencers) play a crucial role in cancer development and recurrence. PcG of proteins is composed of two multimeric protein complexes, that is, the polycomb repressive complex 1 (PRC1) and the polycomb repressive complex 2 (PRC2) [7]. The PRC1 complex includes B cell-specific Moloney murine leukemia virus integration site 1 (BMI1), Mel-18, Mph1/Rae28, M33, Scmh1, and Ring 2, while the PRC2 complex includes Eed, EzH, Suz12, and YY1 [7]. BMI1 is reported to play an important role in self-renewal of stem cells and is associated with a number of human malignancies [2, 5, 8-10]. Recent studies suggest that BMI1 is involved in the initiation of cancer, and targeting BMI1 by gene therapy abolishes chemoresistance in tumor cells [2, 3]. In this review, we summarized (a) the evidences supporting the role of BMI1 in cancer recurrence and chemoresistance, (b) the mechanisms underlying, and (c) the potential approaches that could be used to target BMI1 for cancer therapy.

GENE AND PROTEIN STRUCTURE OF BMI1

Human *BMI1* gene localizes on short arm of chromosome 10 (10p11.23), which comprises 10 exons and 9 introns. The gene encodes a cDNA of approximately 3.4 kb length and a 36.8 kDa protein consisting of 326 amino acids, whereas mouse *Bmi1* gene encodes a protein of 45–47 kDa [2, 5]. With respect to amino acid sequence, a high degree of homology is found between human BMI1 and murine Bmi1 that was the first member of the PcG gene family identified in mammals. BMI1 protein contains a conserved ring finger domain in its N terminal end and a central helix-turn-helix-turn-

Author contributions: H.R.S: data collection, literature collection, and manuscript writing; M.S.: manuscript evaluation and literature verification and writing.

Correspondence: Mohammad Saleem, Ph.D., The Hormel Institute, Department of Laboratory Medicine and Pathology, University of Minnesota-Twin Cities Campus, Minneapolis, Minnesota, USA. Telephone: 507-437-9662; Fax: 507-437-9606; e-mail: msbhat@umn.edu Received October 11, 2011; accepted for publication December 30, 2011; first published online in STEM CELLS *EXPRESS* January 17, 2012; available online without subscription through the open access option. 1066-5099/2012/$30.00/0 doi: 10.1002/stem.1035

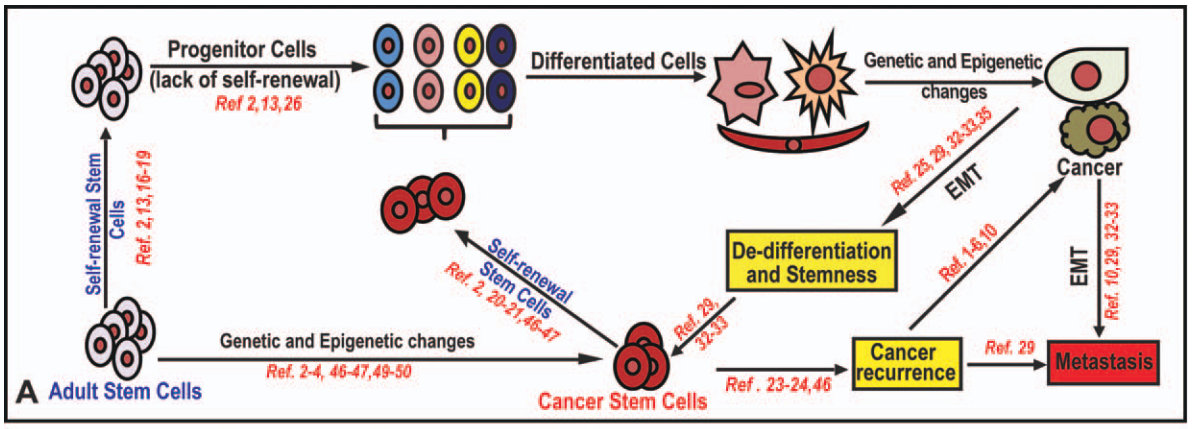

Table showing correlation of BMI1 expression with chemoresistance in different cancer types

Phenotype	Model	Model species	BMI1 status	Therapeutic agents	Function of the therapeutic agents	Chemoresistance	References
Prostate cancer	LNCaP & Du-145	Human	Overexpression	Docetaxel	Pro-apoptotic & anti-proliferative	Yes	3
Pancreatic cancer	Panc-1 (CD44+ cells)	Human	Overexpression	Gemcitabine	Pro-apoptotic & anti-proliferative	Yes	6
Squamous cell carcinoma	SCC-13	Human	Overexpression	EGCG	Pro-apoptotic & anti-proliferative	Yes	11
Normal cell	Keratinocytes	Human	Overexpression	Okadaic acid	Pro-apoptotic	Yes	12
Ovarian	Cancer Stem Cells	Human	Overexpression	Cisplatin and Paclitaxel	Pro-apoptotic & anti-proliferative	Yes	23
Ovarian cancer	A-2780, OCCAR-5 OV-202, OV-167, and CP-70	Human	Overexpression	Cisplatin	Pro-apoptotic & anti-proliferative	Yes	37
Glioma cells	U87MG, U251MG SNB19	Human	Overexpression	Doxorubicin & BCNU	Pro-apoptotic & anti-proliferative	Yes	38
Ovarian cancer	CP-20 mouse model	Mouse	Silenced	Cisplatin	Pro-apoptotic & anti-proliferative	No	37

B

Figure 1. Role of BMI1 in malignant transformation of stem cells into cancer stem cells and chemoresistance. **(A):** Pictorial diagram representing role of BMI1 during cellular events associated with the malignant transformation of stem or differentiated cells into cancer stem cells. The numerical number given on each arrow within the figure represents the reference number cited in the manuscript. **(B):** Table showing correlation of BMI1 expression with chemoresistance in different cancer types assessed in in vitro and in vivo models. Abbreviations: BCNU, Bis-chloroethylnitrosourea; BMI1, B-cell-specific Moloney murine leukemia virus integration site 1; EGCG, epigallocatechin-3-gallate; EMT, epithelial–mesenchymal transition.

helix-turn motif (H-T-H-T), which is essential for inducing telomerase activity [2, 5]. BMI1 contains two nuclear localization signals, KRRR and KRMK.

BMI1 has a ubiquitous pattern of expression in almost all tissues and its expression levels are observed to be high in the brain, esophagus, salivary gland, thymus, kidney, lungs, gonads, placenta, blood, and bone marrow [5]. Balasubramanian et al. [11] has reported the expression of BMI1 in basal and suprabasal keratinocytes. BMI1 is reported to be present in epidermal layers but not in dermis [12].

BMI1 in Normal Stem Cells

Stem cells are of two types (a) embryonic stem cells (ESCs) and (b) adult stem cells (ASCs). ESCs are pluripotent stem cells capable of developing into different cells, however, ASCs maintain and repair their resident tissues in adult organisms. Thus, self-renewal, differentiation, and prevention of senescence of ASCs are critical for tissue homeostasis. BMI1 plays crucial role for self-renewal and differentiation of leukemic stem and progenitor cells [13 and references therein]. BMI1 has also been reported to prevent senescence and immortalize cells through the activation of telomerase [8, 14]. It is reported that Bmi1 plays a crucial role during proliferation of normal stem and progenitor cells derived from fetal liver [13]. Hosen et al. [15] showed that the expression of BMI1 is high in primitive hematopoietic stem cells (HSCs) and is decreased when HSCs are differentiated into a particular lineage. The self-renewal and maintenance of HSCs and neural stem cells (NSCs) were reported to depend on the level of BMI1 protein [8, 16]. These studies suggest a strong correlation of BMI1 with the differentiation and growth of stem cells [15, 16]. BMI1 is reported to play a crucial role during the self-renewal and maintenance of prostate, intestinal, lung epithelial and bronchioalveolar stem cells [17–19].

BMI1 and CSCs

Over the past two decades, evidence has emerged to suggest that cancer could be considered as a stem cell disease and molecular mechanisms governing stem cell self-renewal are subverted during tumorigenesis to maintain cancerous growth (Fig. 1A, 1B) [2]. CSCs were first identified from the blood of patients with acute myeloid leukemia (AML) by Lapidot et al. in 1994 [4]. The CSC theory assumes that both primary and metastatic tumors develop from a small population of cancer cells possessing the characteristics of self-renewal and multipotency and are responsible for initiation and maintenance of tumors (Fig. 1A) [20, 21]. Additionally, CSCs can give rise to wide variety of differentiated cancer cells that comprise the

bulk of the tumor and provide the basis of tumor heterogeneity [20–22]. However, the stability of the CSC phenotype has not yet been completely understood [22]. Published reports suggest that CSCs are responsible for cancer recurrence after therapy and that this property of CSCs is attributed to the activation of different molecules including BMI1 [23–25].

BMI1 expression is frequently upregulated in various types of human cancers [1-2, 23-27]. There are reports that BMI1 acting as an epigenetic modifier protein is involved in the maintenance of CSCs [23, 25]. It is noteworthy that BMI1 is highly enriched in CSCs, however, all BMI1-expressing cells are not CSCs. BMI1 is coexpressed with other stem cell markers (CD133 and CD44) in CSCs [1, 6, 7, 23-26].

Aberrant BMI1 expression is reported in many CSC population. Bmi1 has been reported to be highly expressed in $CD133^+$ murine liver CSCs and play a role in maintenance of hepatic stem/progenitor cells [26]. Zhang et al. [23] observed that ovarian CSCs exhibit higher BMI1 levels than differentiated tumor cells. BMI1 has been shown to be involved in the regulation of CSCs from type-I neuroblastoma [9]. BMI1 was reported to regulate the self-renewal of CSCs by controlling their specific lineage commitment in an expression-dependent manner [9]. AML is a type of cancer in which the bone marrow makes abnormal myeloblasts, red blood cells, and platelets [13]. The proliferation of leukemic stem cells (LSCs) in a mouse model of AML was reported to be promoted by Bmi1 [13]. Bmi1-expressing LSCs were able to induce leukemia when transplanted into irradiated mice, whereas *Bmi1*-null LSCs exhibited limited proliferative potential and were unable to induce disease [13]. This study suggested the critical role of Bmi1 in proliferation of CSCs in leukemia [13]. Medulloblastoma is a type of brain tumor that originates from progenitor cells residing in the external cerebellum. Role of BMI1 in medulloblastoma can be ascertained from the fact that knockdown of *BMI1* in progenitor cells caused suppression in the proliferation and development of disease [27]. These studies suggest that the presence of BMI1 plays an important role in the proliferation of stem cells involved in tumorigenesis.

Different cell types that express BMI1 (such as endothelial cells, mesenchymal stem cells [MSCs], along with CSCs) reside within the tumor microenvironment [20, 28, 29]. The communication between CSCs and other cell types within tumor microenvironment plays an important role in invasion and therapeutic resistance [20, 28, 29]. Each established cell population within tumor exhibit a unique molecular marker that identifies and distinguishes it from other cell types [20-21, 28]. For example, MSCs express aldehyde dehydrogenase 1 (ALDH1) among breast CSCs population [20]. However, there is possibility that unique parental marker/trait still persists in cells that are in a stage of phenotypic transition such as mesenchymal transition [20, 28]. This also holds true with CSCs. A comprehensive discussion on this topic is beyond the scope of the theme of current manuscript.

BMI1, Self-Renewal and Cell Cycle

BMI1 controls self-renewal and cell cycle by regulating the tumor suppressor proteins p16INK4a and p14ARF in cells [8, 14]. BMI1 has been shown to activate the self-renewal ability of NSCs [16]. Recently, Dong et al. [30] demonstrated that loss of BMI1 in endometrial cancer cells reduces expression of stemness genes SOX-2 and KLF4 suggesting that BMI1 is required for regulation of stemness of this cell type.

The p16INK4a protein inhibits binding of Cyclin D to CDK4/6, resulting in the (a) suppression of retinoblastoma (RB) activity and (b) induction of cell cycle arrest [8, 31]. p19Arf (a homolog of human p14ARF) induces p53 and causes cell cycle arrest [8, 16, 31] (Fig. 2A, 2B). BMI1 promotes cell proliferation by suppressing p16INK4A/RB and/or p14ARF/MDM2/p53 tumor suppressor pathways [31]. The absence of BMI1 is reported to relieve the repression of the *INK4a* and resulting in the expression of p16INK4a and p14ARF. Data accumulated so far suggest that BMI1 abolishes cell cycle check points p16/p14 in various cell types (which exhibit different rates of growth/cell cycle kinetics) [7]. We speculate that this holds true for CSCs too. However, the possibility is that BMI1 could not be a sole factor deciding the fate of cells. Although BMI1 is present in CSCs, there is possibility that different subpopulation among CSCs (such as quiescent CSCs) exhibit different rate of growth. This could be possible due to the presence of factors other than BMI1 [18].

BMI1, Epithelial–Mesenchymal Transition and CSCs

The epithelial–mesenchymal transition (EMT) is a key developmental program that is often activated during cancer development [32, 33]. The occurrence of EMT in cancer cells may lead to the number of changes including loss of polarity and epithelial cell markers, loss of contact inhibition, reorganization of the actin cytoskeleton, remodeling of extracellular matrix components, gain of mesenchymal phenotypes along with genetic/epigenetic modifications of different genes, and persistent activation of different growth factors [32, 33]. Published reports suggest a direct link between the EMT and the gain of MSC-like properties [32, 33]. Raimondi et al. [33] reported that the induction of EMT program does not only allow cancer cells to disseminate from the primary tumor but also promotes their self-renewal capability. The sustained stimulation of growth factors may result in an upregulation of diverse gene products in CSCs and their differentiated progenies during the EMT process [32, 33]. Experimental evidence revealed that EMT is involved in anticancer drug resistance [32]. Thus, identification of molecular events that regulate EMT could lead to the development of a new therapeutic approach to suppress growth of CSCs. Song et al. [25] demonstrated that ectopic expression of BMI1 in normal nasopharyngeal epithelial cells is sufficient to cause EMT. Furthermore, this study showed that BMI1 induces EMT by targeting the tumor suppressor PTEN [25]. This in vitro observation was consistent with a cohort of human biopsy samples where an inverse correlation between BMI1 and PTEN was observed [25]. Recently, Yang et al. [29] showed that BMI1 is essential for EMT during tumor development in head and neck cancer patients. This study showed that increased levels of BMI1 were correlated with the worst prognosis in patients with head and neck cancer [29]. The molecules which are frequently altered in cancer cells during the EMT process are E-cadherin, N-cadherin, Vimentin, Tenascin C, NF-κB, SLUG, TWIST, SNAIL, β-Catenin, and CXCR4 [32, 33]. Collectively, these molecules are thought to contribute to the metastatic phenotypes of CSCs and enhance resistance to radiotherapy and chemotherapy [32, 33]. It has been reported that normal human mammary epithelial cells adopt a mesenchymal phenotype and exhibit stem cell-like properties upon expression of SNAIL and TWIST [32]. TWIST is reported to inhibit the senescence inducer proteins (p16 and p21) and co-operates with activated rat sarcoma (RAS) to trigger EMT [32]. Induction of SLUG is known to suppress E-cadherin, which results in the promotion of EMT [34]. Interestingly, $CD133^+$ breast

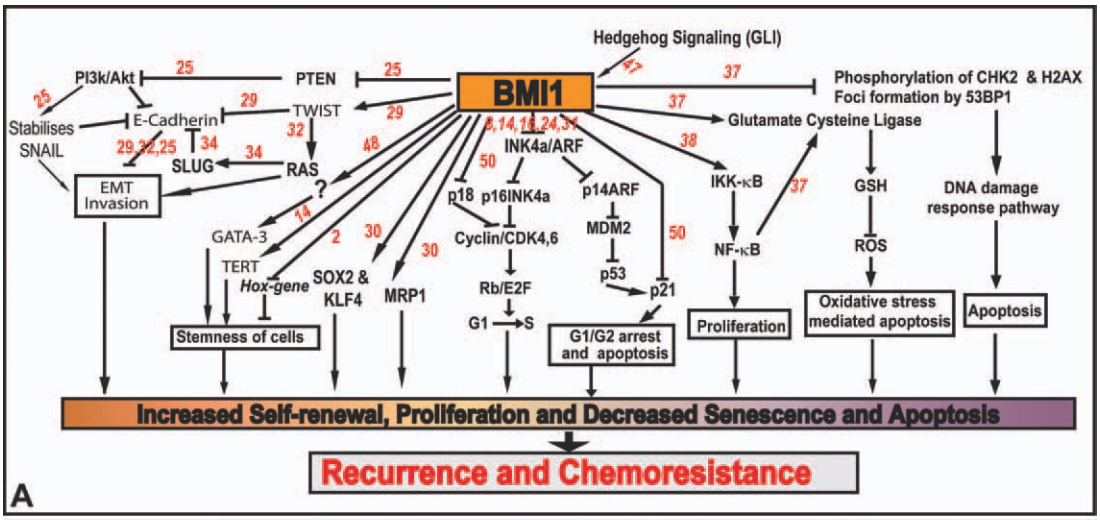

Table showing the published literature that formed the basis of model as represented in Fig. 2A

References	Reference number	References	Reference number
Cao et al., [2011]	2	Jacobs et al., [1999]	31
Park et al., [2004]	8	Thiery et al., [2009]	32
Dimiri et al., [2002]	14	Schmidt et al., [2005]	34
Molofsky et al., [2005]	16	Wang et al [2011]	37
Song et al., [2006]	24	Li et al., [2010]	38
Song et al., [2009]	25	Liu et al., [2006]	47
Yang et al., [2010]	29	Hosokawa et al., [2006]	48
Dong et al., [2011]	30	Facchino et al., [2011]	50

B

Figure 2. Role of BMI1 in cancer recurrence and chemoresistance. **(A):** Flowchart represents role of BMI1 and its interacting proteins during self-renewal, proliferation, and chemoresistance of cancer cells. **(B):** Table showing the published literature that formed the basis of model as represented in (A). The numerical number given on each arrow within the figure represents the reference number cited in the manuscript. ⊣ Represents inhibition and → represents activation. Abbreviations: BMI1, B-cell-specific Moloney murine leukemia virus integration site 1; EMT, epithelial–mesenchymal transition; GSH, reduced glutathione; IKK, IKB kinase; NF, nuclear factor; ROS, reactive oxygen species.

CSCs that express SLUG are also found to express high BMI1 [35]. BMI1 in co-operation with TWIST1 was reported to promote cancer dedifferentiation and metastasis [29]. Keeping in view that (a) EMT and stemness are interlinked processes, (b) EMT and stemness processes confer chemoresistance to tumor cells, and (c) BMI1 plays role in both EMT and stemness processes, the importance of BMI1 in chemoresistance as a major factor is further strengthened.

BMI1 and Chemoresistance: Preclinical Evidences

The inability of tumor cells to undergo apoptosis in response to chemotherapy poses a selective advantage for tumor progression, metastasis, and resistance to therapy. BMI1 has been reported to be associated with the protection of tumor cells from apoptosis (Fig. 1B). Cui et al. [9] showed that the ectopic expression of BMI1 rescues keratinocytes from stress-induced apoptosis. Bmi1 knockdown was observed to increase the apoptosis in lymphocytes in spleen and thymus in an animal model [36]. Zhang et al. [23] observed that ovarian CSCs exhibiting high BMI1 levels have increased resistance to Cisplatin and Paclitaxel. Crea et al. showed that BMI1 silencing significantly enhanced the antitumor efficiency of Docetaxel against prostate cancer cells. BMI1 (by modulating antioxidant machinery) was observed to allow prostate tumor cells to survive after chemotherapy [3]. Examination of clinical datasets revealed a positive correlation of BMI1 and antioxidant gene expression in patients exhibiting chemoresistance [3]. Recently, Wang et al. [37] reported that BMI1 is involved in chemoresistance of ovarian cancer cells, and targeting BMI1 by gene therapy sensitizes tumor cells to Cisplatin chemotherapy. Modulation of reduced glutathione (GSH) and CHK2 and H2AX molecules by BMI1 was reported as the underlying mechanism for chemoresistant behavior of ovarian tumor cells [37]. BMI1 silencing was found to reduce intracellular GSH levels and sensitize cancer cells to Cisplatin [37]. It is noteworthy that Cisplatin-induced apoptosis in such cell was found to be mediated by reactive oxygen species (ROS) generation [37]. Recent studies showed that overexpression of BMI1 rescues tumor cells from the apoptosis induced by Okadaic acid and Epigallocatechin-3-gallate, well-known apoptotic agents [11, 12]. Interestingly, artificial introduction of BMI1 in chemosensitive tumor cells was observed to confer chemoresistance in such cells [11]. Yin et al. [6] showed that $CD44^+/CD24^+$ pancreatic cancer cells expressing high levels of BMI1 exhibit chemoresistance to Gemcitabine treatment. Li et al. [38] reported that BMI1 by activating NF-κB significantly inhibits Doxorubicin-, BCNU-, and UV irradiation-induced apoptosis in glioma cells. Recently, we observed that the reduction of BMI1 protein levels by gene therapy abolishes chemoresistance in prostate carcinoma cells (Siddique et al., unpublished data). Taken together, these studies support the role BMI1 plays in conferring chemoresistance to tumor cells.

BMI1 and Chemoresistance: Clinical Evidences

The clinical significance of BMI1 in chemoresistance and its correlation with therapy failure in several cancer types has been established [5, 9, 10, 39–40]. BMI1 was found to be one of the key regulatory factors determining a cellular phenotype captured by the expression of a death-form-cancer signature in a broad spectrum of therapy-resistant cancers, including five epithelial (prostate, breast, lung, ovarian, and bladder cancers) and five nonepithelial (lymphoma, mesothelioma, medulloblastoma, glioma, and AML) malignancies [39]. Glinsky et al. [39] described a conserved BMI1-driven pathway of 11-gene signature which defines stemness of highly invasive tumors of multiple tissue origin and correlation with therapy failure. High level of BMI1 in tumors was reported to be positively correlated with poor prognosis in nasopharyngeal carcinoma patients [24]. BMI1 was identified as predictive factor for overall survival in patients with head and neck squamous cell carcinomas (HNSCC) [41]. BMI1 levels were observed to be increased in 79% of HNSCC patients, and a positive correlation was observed between BMI1 levels and lack of response to radiotherapy or chemotherapy [41]. Van Kemenade et al. [42] reported that poor outcome and aggressive tumor behavior were correlated with high BMI1 levels in patients with non-Hodgkin B-cell lymphomas and nasal pharyngeal carcinoma. Li et al. [38] showed that BMI1 was upregulated in 93.9% glioma specimens from 297 patients. This study showed that BMI1 expression was inversely correlated with survival time of glioma patients and positively correlated with the poor prognosis of the disease [38]. Mihic-Probst et al. [10] studying 329 melanoma patients reported that high expression of BMI1 in 64% of primary and 71% metastatic melanoma was associated with clinical progress of the disease. Recent reports show a correlation between BMI1 levels and recurrence cum survival of disease in tongue cancer, oropharyngeal squamous cell cancer, and non-small cell lung cancer (NSCLC) patients [5, 43, 44]. Disease-free survival for stage I and II of NSCLC patients who had received adjuvant therapy was reported to be better in BMI1-negative patients than BMI1-positive counterparts [44]. We observed a stage-dependent increase in human prostatic tumors and decreased chemoresistance in cells exhibiting reduced BMI1 levels (Siddique et al., unpublished data). Collectively these studies also suggest that BMI1 might be applicable as predictive markers of therapy during the follow-up of patients undergoing chemotherapy.

Molecular Mechanisms of BMI1-Induced Chemoresistance

Chemoresistance has been reported to be caused by the aberration of several molecular pathways in tumor cells. CSCs have been shown to display chemoresistance through (a) modulation of DNA repair machinery, (b) ATP-binding cassette (ABC) multidrug resistance, (c) quiescence, and (d) upregulation of antiapoptotic genes [45]. Emerging evidences support the notion that BMI1 is an important molecule in the process of chemoresistance. However, the precise mechanism of BMI1 on the regulation of chemoresistance in tumor cells is not completely understood. As presented in Figure 2, BMI1 is reported to modulate several molecular pathways within the cells. BMI1 has been shown to induce its effect at epigenetic as well as genetic level [7, 13, 46]. It is believed that chromatin modifications induced by PcG proteins (including BMI1) create an obstacle to transcription factors and RNA polymerase binding [46]. BMI1 has been shown to modulate chromatin by (a) forming a complex with methylated Lys_{27} of H3 and (b) catalyzing the ubiquitinylation of histone H2A [7, 46]. The co-operation between the Eed complex (that modifies chromatin by recruiting histone deacetylases) and BMI1 complex leads to the silencing of target gene expression [7, 46]. BMI1 induces immortalization of cells by downregulating the p16INK4a and p14ARF [8, 16]. Huber et al. [5] reported a correlation between low expression of p16 and high expression of BMI1 in human cancer patients. It is reported that the cooperation of BMI1 with c-MYC results in induction of telomerase activity and downregulation of *INK4a*/ARF [36].

Sonic Hedgehog (SHH) pathway is reported to play a role in the self-renewal of breast stem/progenitor cells [47]. SHH-activated mammosphere formation is reported to be mediated by BMI1 [47]. BMI1 is reported to regulate intracellular GSH levels by modulating glutamate cystine ligase, which is also positively regulated by Nrf-1 and nuclear factor κB (NF-κB) [37]. It is noteworthy that BMI1 expression was reported to be positively associated with activity of Nrf-1 and NF-κB in glioma cells [38]. BMI1 is reported to occupy the *PTEN* locus and downregulates *PTEN* expression [25]. Occupancy of BMI1 on *PTEN* locus results in the activation of Phosphatidylinositol 3-kinases/protein kinase B (PI3K/AKT) pathway, stabilization of SNAIL, and downregulation of E-cadherin. BMI1 directly occupies the promoters of CDH1 (which encodes E-cadherin) and *INK4a* [25]. Lee et al. [12] showed that BMI1 influences cell proliferation by increasing the expression levels of cyclin-dependent Kinase 2, 4 (CDK2, CDK4), and Cylin D1. BMI1 is reported to regulate stability of GATA binding protein 3 (GATA3), a transcription factor that is involved in Th2 cell development and differentiation [48]. Recently, Dong et al. [30] demonstrated that loss of BMI1 in endometrial cancer cells reduces expression of drug resistance gene MRP1, suggesting that BMI1 is required for the drug resistance. Quiescent nature of CSCs represents an inherent mechanism that at least partially explains chemotherapy resistance and recurrence in post-therapy in cancer patients [18, 20, 30]. Recent study by Tian et al. [18] suggest that Bmi1 plays an important role in the maintenance and growth of quiescent cells. Bmi1-expressing quiescent cells were shown to contribute to the generation of epithelial cells of intestine [18]. It is noteworthy that this effect of BMI1 was observed under conditions when proliferative cells were not sufficient and BMI1 expressing-quiescent cells were found to grow into tissue [18].

BMI1: A Potential Target for Cancer Therapy

CSCs may be eliminated by selectively targeted therapies against BMI1 [49, 50] (Siddique et al., unpublished data). However, it would be much complex to selectively target CSCs without any harmful effects to normal stem cells because normal stem cells and CSCs share the same pathways to maintain their self-renewal capability. It appears that CSCs are more likely to be more dependent on certain putative pathways. In this context, Liu et al. showed that human BMI1 is critical for the short-term survival of cancer cells, and inhibition of BMI1 has minimal effect on the survival of normal cells. These findings provide a foundation for developing a cancer-specific therapy targeting BMI1 [49]. Recently, Facchino et al. showed that glioblastoma multiforme (GBM) stem cells acquire an oncogenic trait by BMI1 overexpression thus distinguishing CSCs from normal stem cells. This situation was observed to render GBM stem cells

more sensitive to BMI1 inhibition than normal stem cells [50]. Based on compelling evidences (which suggest the critical role of BMI1 in growth and proliferation), using BMI1 as a target for anticancer therapy seems an ideal option. Wang et al. successfully tested 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine nanoparticles carrying small inhibitory RNA (siRNA) to target BMI1 and reported an inhibition in the growth of chemoresistant ovarian tumors implanted in a xenograft mouse model [37]. This study showed that gene therapy-induced BMI1 silencing along with Cisplatin completely abrogated ovarian tumor growth [37].

We recently showed that targeted inhibition of BMI1 by adopting gene therapy approach resulted in the reduction in the invasive potential and tumorigenic potential of prostate cancer cells (Siddique et al., unpublished data). We have embarked upon a broad program aimed to evaluate the potential and usefulness of BMI1 as a molecular target for human cancers. We have developed specific BMI1 small molecule inhibitors (Siddique et al., unpublished data), which were observed to inhibit the proliferative potential of prostate, pancreatic and skin cancer cells (Siddique et al., unpublished data).

Conclusions

BMI1 has been reported to be associated with the progression, recurrence, and chemoresistance to the various types of cancer cells. Hence, it is of great clinical value to further understand the molecular mechanism underlying the regulation of BMI1 in CSCs and chemoresistance. This will not only help in understanding the role of BMI1 in the growth of CSCs and chemoresistance but will also provide insights for the establishment of new strategies and effective clinical therapies for the treatment of chemoresistant cancers. Taken together, these studies show that BMI1 has the potential to be developed as a target for therapeutic agents and small molecules efficiently targeting BMI1 offer an option as future anticancer drugs.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflict of interest.

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